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SYNERGISM BETWEEN ONCOGENIC HUMAN HERPES VIRUSES AND CARCINOGENIC CHEMICAL

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FOR THE COMMANDER

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Six chemicals were tested for synergism in promoting in vitro oncogenic transformation of Swiss mouse 3T3 cells by herpes simplex type 2 virus (HSV-2). The test chemicals were N,Nitrosodimethylamine, 1,2-dimethylhydrazine, hydrazine, methylhydrazine, 1,1-dimethylhydrazine, and N-phenyl-alpha-naphthylamine. 1,2-dimethylhydrazine (symmetrical dimethylhydrazine, SDMH) enhanced the level of oncogenic transformation approximately two-fold. None of the other test chemicals enhanced HSV-2 transformation at the concentrations tested. The significance of these data is somewhat uncertain at this point, but certainly follow-up studies should be conducted to confirm the apparent enhancement of viral transformation by SDMH.

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PREFACE

F. Brent Johnson, Ph.D., is an Associate Professor of Microbiology in the Microbiology Department, Brigham Young University. While doing the research presented in this report, Dr. Johnson was assigned to the Toxicology Branch, Toxic Hazards Division, Aerospace Medical Research Laboratory as a Research Associate under the USAF-ASEE Summer Faculty Research Program. Capt Richard A. Davis acted as his Research Colleague during the program. The research was performed in the Cytology Laboratory of the Toxic Hazards Research Unit in support of Project 6302 "Toxic Hazards of Propellants and Materials", Task 630201 "Toxicology of Propellants and Materials", Work Unit 63020104 "Toxicological Screening of Toxic Chemicals and Materials Used in Air Force Operations".

INTRODUCTION

Herpes simplex viruses have been implicated in a variety of diseases ranging from the fever blister to neoplastic disease. Herpes simplex type 1 (HSV-1) is usually isolated from oral or facial lesions, while herpes simplex type 2 (HSV-2) is most commonly isolated from genital lesions. The virus has a propensity for infection of the mucous membranes or the cutaneous-mucous membrane junctures; however, the virus can remain latent, presumably within neurons, for indefinite periods of time. During childhood the most common result of primary herpes infection is gingivostomatitis. This common infection is often subclinical. It is presumed to be responsible for the seroconversions which result in the prevalence of HSV antibodies found in the population after the early childhood years. A sequel to primary infection is the establishment of latent HSV infection which, when activated, causes fever blisters and cold sores. HSV is also responsible for more serious infections of a generalized nature, which include disseminated infections of the newborn, meningitis, or encephalitis.

Recurrent infections with HSV are very common. Studies have been conducted which show that between 40 and 90 percent of the adult population are serologically positive and are, therefore, subject to periodic cutaneous and mucosal recurrences involving oral or genital sites (Buddingh et al., 1953; Smith and Peutherer, 1967). Although the lesions are usually more of a nuisance than they are debilitating, a number of patients exhibit either such severe or very frequent episodes that considerable medical treatment is required (Berg, 1955; Douglas et al., 1969; Nash and Foley, 1970; Ross and Stevenson, 1961). Furthermore, the neoplastic potential of HSV-2 has been well established, as has the association of this virus with venereal disease, and with cervical cancer by seroepidemiological studies (Nahmias et al., 1973). In vitro transformation by HSV-2 has been demonstrated in rat embryo cells at 42°C (Darai and Munk, 1976), and in mouse 3T3 cells with ultraviolet irradiated virus (Duff and Rapp, 1975).

The purpose of the present study was to determine by in vitro assays whether herpes viral oncogenic transformation is enhanced by chemical carcinogens. It is possible that synergistic effects result in a much higher frequency of transformation when the target cell is exposed to both agents, or, on the other hand, chemical and viral transformation may be completely separate events. The results of this study indicate possible transformation enhancement by 1,2-dimethylhydrazine and no apparent enhancement by the other test chemicals.

MATERIALS AND METHODS

Cells. HEp-2 cells were brought by the author from his own laboratory at Brigham Young University. They were grown in Basal Medium Eagle's (modified) with Earle's salts (BME) supplemented with 10% fetal calf serum, 0.22% sodium bicarbonate, glutamine (4 mM), penicillin (100 units per ml), and streptomycin (100 microgram per ml). The HEp-2 cells were passaged 1:5 at four day intervals and were maintained in 75 cm^2 plastic tissue culture flasks.

The 3T3 cells (Swiss albino mouse, contact inhibited fibroblasts) were obtained from the American Type Culture Collection (CCL 92) at passage number 118. They were grown in Earle's BME (described above) in 75 cm^2 plastic tissue culture flasks. They were subcultured every three days at a dilution of 1:3.

Virus. HSV-2 (strain 333) was brought from the author's laboratory and was originally obtained from Dr. Byron K. Murray, Medical College of Virginia, Virginia Commonwealth University. A fresh stock of the virus was prepared in HEp-2 cells, freeze-thawed one time then titrated in HEp-2 cells in 60 mm petri dishes using conventional techniques and methylcellulose overlays. The virus titer was 3.5×10^7 plaque forming units (pfu) per ml. Stock vials of the virus were stored in liquid nitrogen and were never refrozen or reused after once thawing.

Inactivation of HSV-2 by ultraviolet light. Virus stocks for transformation studies were inactivated by UV irradiation at a calculated dose of 60 ergs/s/mm^2. The appropriate virus concentration (1.5 ml) was placed in a 60 mm plastic petri dish, the lid removed, and exposed to the UV source for two minute intervals interspaced with 30 seconds of agitation.

Transformation assays. These assays were carried out by methods similar to those reported by Duff and Rapp (1975). 3T3 cells (1×10^6 cells contained in one ml) were infected with UV-treated HSV-2 (5×10^6 pfu) while in suspension with gentle shaking. One hour after infection, each virus-cell mixture was distributed among four 60 mm plastic petri dishes each containing 5 ml of modified BME medium containing 10% fetal calf serum and 0.22% sodium bicarbonate. The cultures were incubated at 36.5°C in an atmosphere of 5% CO_2. The medium was changed on each culture every three days. The cultures were stained with Giemsa stain 28 days after initial infection, and the transformed foci were identified and counted microscopically. The foci that were scored positive for transformation exhibited loss of contact inhibition with the cells oriented in a criss-cross pattern.

In the experiments which incorporated the test chemicals, the medium was removed six hours after plating the infected cells, or the mock-infected cells, and replaced with 5 ml of media per plate containing the appropriate concentrations of the test chemicals. After addition of the test chemicals to the media, they were filtered through 0.2 micron membrane filters to restore sterility. These cultures were observed daily for cytopathic effects (CPE). At the end of four days, the media were replaced with fresh medium (BME) containing no test chemicals. The cultures were maintained for 28 days, the medium changed every four days, and were stained with Giemsa stain and observed microscopically for foci of transformed cells.

RESULTS

Determination of the optimum UV inactivation dose. In order to determine the optimum conditions for formation of foci of 3T3 cells transformed by HSV-2,

the virus was irradiated with UV light for 2, 4, 6, 8, 10, 12 and 14 minutes at 60 ergs/s/mm^2. Cells infected with virus irradiated for these various times were plated and observed for transformed foci. The results are shown in Table 1. It will be noted that the viral-induced CPE diminished with increasing exposure to the ultraviolet light. The only detectable transformed foci appeared in the cultures infected with virus exposed for 2, 4, and 6 minutes. Thus, more than 75% of the cells were killed outright by the virus and the transformants developed from the survivors. The cells infected with virus exposed for longer than six minutes did not develop any detectable transformed foci, presumably because of too much damage in the viral transforming gene(s). At approximately two weeks into the assay, some viral "Breakthrough" was noted when small areas of the confluent cell sheet began to degenerate because of viral CPE, but they recovered and were nearly all confluent at the termination of the experiment. An exposure of four minutes was chosen for the subsequent transformation assays because a maximum number of foci were noted at this time and there was near a minimum amount of breakthrough.

Transformation in the presence of the test chemicals. Cells in suspension were infected, at a multiplicity of infection of five, with UV-irradiated HSV-2. The infected cells and mock-infected cells were plated, the cells allowed to attach, then exposed to media containing various concentrations of the test chemicals. The results are shown in Tables 2 to 7. At the concentrations tested, hydrazine and methylhydrazine were highly toxic to the cells. Toxicity was judged by the development of CPE, that is, the rounding and detachment of the cells. The normal cell is a spindle-shaped fibroblast. Cells affected by viral CPE are rounded, swollen, and detach from the plastic vessel, often in clumps. Cells affected by the chemicals were rounded and shrunken. They detached from the surface as individual, single cells. UDMH was only moderately toxic and N-phenyl-alpha-naphthylamine was toxic only at a very high concentration. N,nitrosodimethylamine and SDMH were non-toxic at the concentrations tested.

When the cultures were stained and examined for transformed foci, no foci were found either in the cell controls or in any of the cultures treated with the test chemicals in the absence of virus. Foci were detected only in those cultures infected with virus, either alone or in combination with the test chemical (Tables 2 to 7). It will be noted that, at the concentrations tested, none of the test chemicals enhanced the frequency of transformation except SDMH which gave an apparent two-fold enhancement. At concentrations to 5 μg/ml and 1 μg/ml, SDMH decreased markedly the level of transformation expected, based on the control value. This interference with transformation may be explained by possible genetic damage to the viral genome. As the dosage levels of SDMH were further diminished, a peak level of transformed foci was obtained at 0.5 μg/ml. It is not yet known whether these data are reproducible, but it should be emphasized that this approximate two-fold enhancement resulted in numbers of transformed foci that were well outside of the range of the foci found in the virus control cultures.

The virus controls included in the UDMH experiment (Table 6) developed an unusually low number of foci; hence, the level of transformation noted in the cultures containing 0.05 µl/ml of UDMH was not interpreted as enhancement since this level was within the levels of most of the viral control assays.

DISCUSSION

The major question examined in this study centered on the possibility that mutagenic chemicals can act in concert with an oncogenic virus in causing cell transformation. An unexpected side result was the development of chemical-induced toxicity, because the dosage levels tested were previously found to be non-toxic for WI-38 cells and L5178Y mouse cells (Brusick and Matheson, 1976a; 1976b; 1976c). Nevertheless, in general, hydrazine and its derivatives that were found to possess highest toxicity to 3T3 cells have previously been shown to possess greatest toxicity in in vivo assay systems.

If confirmation of the apparent transformation-enhancement by SDMH is forthcoming, it will be of importance to examine other chemical mutagens for enhancing activity. Thus, it may be of major importance for those persons who have latent or active HSV-2 infections to avoid exposure to such chemicals. Other potentially oncogenic human viruses, such as the adenoviruses, should also be tested for enhancement. Also, a broader range of dosages of hydrazine and methylhydrazine should be tested to obtain data on several dosage levels outside of the toxic range.

An observation made during this study was that virus breakthrough occurred in several of the cultures treated with the test chemicals, but in none of the virus control cultures. It is possible that the chemicals were inhibiting the production or activity of antiviral substances within the cells or promoting repair of the UV-damaged viral genomes, the latter of which seems unlikely. Finally, it is not known why N-nitrosodimethylamine, a known carcinogen, was not active in causing enhancement. It seems apparent that whatever the mechanism of enhancement, it may be, at least in some cases, independent of indiscriminate mutagenic activity.

TABLE 1. Transformation of 3T3 cells by UV-irradiated HSV-2: Optimum UV dose

UV exposure (minutes)	Maximum CPE[a]	Transformed foci per 10^6 cells
2	++++	5
4	++++	9
6	+++	8
8	++	0
10	+	0
12	None	0
14	None	0
Cell Controls (no virus)	None	0

a + = 25% of cells affected.
++ = 50% of cells affected.
+++ = 75% of cells affected.
++++ = 100% of cells affected.

TABLE 2. Effect of N,Nitrosodimethylamine on HSV-2 transformation of 3T3 cells

	Maximum Viral CPE	Maximum Chemical CPE	Confluency Obtained	Transformed foci per 10^6 cells
Cell Controls[a]	0	0	Yes	0
Virus Controls[b]	+++	0	Yes	10
Virus + NDMA				
0.5 µl/ml	+++	0	Yes	8
0.1 µl/ml	+++	0	Yes	11
0.05 µl/ml	+++	0	Yes	8
0.01 µl/ml	+++	0	Yes	4
NDMA Controls[c]				
0.5 µl/ml	0	0	Yes	0
0.1 µl/ml	0	0	Yes	0
0.05 µl/ml	0	0	Yes	0
0.01 µl/ml	0	0	Yes	0

a 3T3 cells exposed to neither virus nor test chemical
b 3T3 cells exposed to UV-irradiated HSV-2
c 3T3 cells exposed to the test chemical; no virus

TABLE 3. Effect of 1,2-dimethylhydrazine on HSV transformation of 3T3 cells

	Maximum Viral CPE	Maximum Chemical CPE	Confluency Obtained	Transformed foci per 10^6 cells
Cell Controls[a]	0	0	Yes	0
Virus Controls[b]	+++	0	Yes	21
Virus + SDMH				
5 µg/ml	+++	0	Yes	1
1 µg/ml	+++	0	Yes	4
0.5 µg/ml	+++	0	Yes	58
0.1 µg/ml	+++	0	Yes	37
SDMH Controls[c]				
5 µg/ml	0	0	Yes	0
1 µg/ml	0	0	Yes	0
0.5 µg/ml	0	0	Yes	0
0.1 µg/ml	0	0	Yes	0

a 3T3 cells exposed to neither virus nor test chemical
b 3T3 cells exposed to UV-irradiated HSV-2
c 3T3 cells exposed to the test chemical; no virus

TABLE 4. Effect of Hydrazine on HSV-2 transformation of 3T3 cells

	Maximum Viral CPE	Maximum Chemical CPE	Confluency Obtained	Transformed foci per 10^6 cells
Cell Controls[a]	0	0	Yes	0
Virus Controls[b]	+++	0	Yes	16
Virus + hydrazine				
0.5 µl/ml	+++	++++	No	-
0.1 µl/ml	+++	++++	No	-
0.05 µl/ml	+++	++++	No	-
0.01 µl/ml	+++	+	Yes	5
Hydrazine Controls[c]				
0.5 µl/ml	0	++++	No	-
0.1 µl/ml	0	++++	No	-
0.05 µl/ml	0	++++	No	-
0.01 µl/ml	0	+	Yes	0

a 3T3 cells exposed to neither virus nor test chemical
b 3T3 cells exposed to UV-irradiated HSV-2
c 3T3 cells exposed to the test chemical; no virus

TABLE 5. Effect of methylhydrazine on HSV-2 transformation of 3T3 cells

	Maximum Viral CPE	Maximum Chemical CPE	Confluency Obtained	Transformed foci per 10^6 cells
Cell Controls[a]	0	0	Yes	0
Virus Controls[b]	+++	0	Yes	11
Virus + MMH				
0.5 µl/ml	+++	++++	No	-
0.1 µl/ml	+++	++++	No	-
0.05 µl/ml	+++	++++	No	-
0.01 µl/ml	+++	+	Yes	8
MMH Controls[c]				
0.5 µl/ml	0	++++	No	-
0.1 µl/ml	0	++++	No	-
0.05 µl/ml	0	++++	No	-
0.01 µl/ml	0	+	Yes	0

a 3G3 cells exposed to neither virus nor test chemical
b 3T3 cells exposed to UV-irradiated HSV-2
c 3T3 cells exposed to the test chemical; no virus

TABLE 6. Effect of 1,1-dimethylhydrazine on HSV-2 transformation of 3T3 cells

	Maximum Viral CPE	Maximum Chemical CPE	Confluency Obtained	Transformed foci per 10^6 cells
Cell Controls[a]	0	0	Yes	0
Virus Controls[b]	+++	0	Yes	3
Virus + UDMH				
0.5 µl/ml	+++	++++	No	-
0.1 µl/ml	+++	+	No	-
0.05 µl/ml	+++	0	Yes	11
0.01 µl/ml	+++	0	Yes	3
UDMH Controls[c]				
0.5 µl/ml	0	++++	No	-
0.1 µl/ml	0	+	Yes	0
0.05 µl/ml	0	0	Yes	0
0.01 µl/ml	0	0	Yes	0

a 3T3 cells exposed to neither virus nor test chemical
b 3T3 cells exposed to UV-irradiated HSV-2
c 3T3 cells exposed to the test chemical; no virus

TABLE 7. Effect of N-phenyl-alpha-naphthlamine on HSV-2 transformation of 3T3 cells

	Maximum Viral CPE	Maximum Chemical CPE	Confluency Obtained	Transformed foci per 10^6 cells
Cell Controls[a]	0	0	Yes	0
Virus Controls[b]	+++	0	Yes	11
Virus + PANA				
50 µg/ml	+++	++++	No	-
10 µg/ml	+++	0	Yes	6
5 µg/ml	+++	0	Yes	4
PANA Controls[c]				
50 µg/ml	0	++++	No	-
10 µg/ml	0	0	Yes	0
5 µg/ml	0	0	Yes	0

a 3T3 cells exposed to neither virus nor test chemical
b 3T3 cells exposed to UV-irradiated HSV-2
c 3T3 cells exposed to the test chemical; no virus

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